

Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl *cis-trans* isomerase

Implications for the immunosuppressive and toxic effects of cyclosporin

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The K_i values of cyclosporins A, G and H for the peptidyl-prolyl *cis-trans* isomerase (PPIase) of liver and heart mitochondria are about 2, 20 and 500 nM respectively. This parallels their profile as inhibitors of non-specific pore opening of mitochondria induced by supraphysiological Ca^{2+} concentrations. The novel immunosuppressant FK-506 gave little inhibition of either process at 5 μM . These data support our previous hypothesis [Halestrap & Davidson (1990) *Biochem. J.* 268, 153–160] that pore opening involves an interaction between matrix PPIase and the adenine nucleotide translocase. It is suggested that this model may help to clarify the mechanism of action of cyclosporin as an immunosuppressant and its toxic effects on the liver and kidney following prolonged therapy.

INTRODUCTION

Cyclosporin A is a widely used immunosuppressive drug which inhibits the activation of T-lymphocytes by a variety of stimuli [1–3]. The drug binds tightly to a 17 kDa cytosolic protein, cyclophilin, now known to be a peptidyl-prolyl *cis-trans* isomerase (PPIase) [4,5]. The mechanism by which inhibition of this enzyme blocks the activation of lymphocyte gene expression is unknown. Cyclosporin A also prevents damage to mitochondria after exposure to supraphysiological Ca^{2+} concentrations, as occurs in reperfusion injury [6–8]. These mitochondria become swollen, leaky and unable to support oxidative phosphorylation as a result of the opening of a non-specific permeability pathway in the mitochondrial inner membrane [6,9]. Pore opening is also modulated by effectors of the adenine nucleotide translocase [6–11], which had led us [8] and others [11] to implicate this protein in pore formation. We have demonstrated that both rat liver and heart mitochondria possess about 120 pmol of PPIase/mg of protein, and that inhibition of this enzyme by cyclosporin A parallels the inhibition of mitochondrial swelling induced by high Ca^{2+} concentrations. This led us to propose that under conditions of Ca^{2+} overload the isomerase can bind to the adenine nucleotide translocase, causing it to open into a non-specific channel [8]. In this paper we show that the K_i values of cyclosporins A, G and H for the PPIase of liver and heart mitochondria are about 2, 20 and 500 nM respectively, which parallels their profile as inhibitors of pore opening. The novel immunosuppressant FK-506, which is known to be a potent inhibitor of a quite separate cytosolic PPIase with a K_i of less than 5 nM [12–16], gave little inhibition of either process at 5 μM . These data may help to clarify the mechanism of action of cyclosporin as an immunosuppressant and its toxic effects on the liver and kidney following prolonged therapy [1,2].

EXPERIMENTAL

Materials

Cyclosporin analogues and FK-506 were generously provided by Sandoz Ltd., Basel, Switzerland, and Fujisawa Pharmaceutical

Co. Ltd., Osaka, Japan, respectively. The sources of all other chemicals and biochemicals and the methods used to prepare rat liver and heart mitochondria have been described previously [8,16].

Methods

The Ca^{2+} -mediated opening of the non-specific pore was studied by measuring the decrease in light scattering of de-energized mitochondria incubated in iso-osmotic KSCN, as described previously [8]. Liver or heart mitochondria (10–15 mg of protein) were added to 7.4 ml of buffer containing 150 mM-KSCN, 10 mM-Mops, 5 mM-Tris, 0.5 μg of rotenone/ml and 0.5 μg of antimycin/ml, pH 7.2, at 30 °C. After mixing, 3.5 ml portions of the suspension were added to both sample and reference cuvettes of a split-beam spectrophotometer. Swelling was initiated by addition of 250 μM - CaCl_2 to the sample cuvette, and the decrease in light scattering (A_{520}) was monitored continuously with an on-line computer.

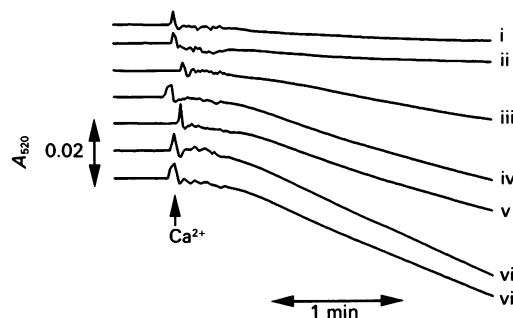


Fig. 1. Effect of cyclosporin analogues and FK-506 on Ca^{2+} -induced mitochondrial swelling

Swelling was measured as described in the Experimental section. Cyclosporin (Cy) or FK-506, dissolved in dimethyl sulphoxide, were added to the mitochondrial incubations at the following concentrations: none (trace vii), 5 μM -FK-506 (vi), 5 μM -CyH (v), 100 nM-CyG (iv), 100 nM-CyA (iii), 5 μM -CyG (ii) and 5 μM -CyA (i).

Abbreviation used: PPIase, peptidyl-prolyl *cis-trans* isomerase.

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Table 1. K_i values for inhibition by cyclosporin analogues of Ca^{2+} -induced mitochondrial swelling and PPIase activity

K_i values are given as means \pm S.E.M. of the numbers of experiments shown in parentheses. Each experiment involved a different preparation of mitochondria or matrix fraction, and initial rates of Ca^{2+} -induced swelling or isomerase activity were measured with at least eight cyclosporin and two protein concentrations. K_i values were determined by non-linear regression analysis to the equation for inhibition by tight-binding inhibitors [8], assuming that for both heart and liver mitochondria the number of cyclosporin-binding sites/mg of total mitochondrial protein and matrix fraction are 120 and 400 pmol respectively [8].

Inhibitor	K_i (nM)			
	Liver		Heart	
	Swelling	Isomerase	Swelling	Isomerase
Cyclosporin A	4.4 ± 0.8 (8)	2.6 ± 0.5 (4)	13.7 ± 2.0 (8)	3.8 ± 1.2 (3)
Cyclosporin G	101.0 ± 9.2 (11)	21.2 ± 2.0 (5)	105.0 ± 14.2 (4)	20.8 ± 1.8 (4)
Cyclosporin H	> 500	> 500	> 500	> 500
FK-506	> 5000	> 5000	> 5000	> 5000

PPIase activity was measured in mitochondrial matrix fractions prepared from liver mitochondria by digitonin treatment and sonication [8]. Assay of PPIase at 10 °C was performed as described previously [8]. In outline, 3.5 ml of buffer (35 mM-Hepes, pH 7.8, containing 26 mg of chymotrypsin/ml) was added to the sample cuvette of a split-beam spectrophotometer. In order to balance the signal, the reference cuvette contained a previous assay that had reached completion. When required, the cyclosporin analogue or FK-506 and matrix extract were added to the sample cuvette which was constantly stirred. A_{390} was monitored by an on-line computer, and after 30 s the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-nitroanilide (dissolved in dimethyl sulphoxide, was added to the sample cuvette through an injection port to give a final concentration of 35 μM . Collection of data was continued until the reaction reached completion.

RESULTS

Effects of cyclosporin analogues on Ca^{2+} -dependent mitochondrial swelling

When de-energized mitochondria are incubated in iso-osmotic KSCN, opening of the non-specific pore can be initiated by addition of 250 μM - Ca^{2+} [8,16]. Entry of KSCN into the matrix through the pore is associated with osmotically driven water uptake, and the consequent swelling is detected as a decrease in light scattering at 390 nm. The data of Fig. 1 show the inhibition of this swelling by various cyclosporin analogues. At 5 μM , both cyclosporin A and cyclosporin G inhibited swelling completely (traces i and ii), while cyclosporin H gave only about 50% inhibition (trace v) and FK-506 was without effect (trace vi). At 100 nM it was clear that cyclosporin A was a more potent inhibitor than cyclosporin G (traces iii and iv), whereas cyclosporin H was without effect (not shown). We have performed a series of such experiments using both heart and liver mitochondria and cyclosporin A and G concentrations in the range 0–1 μM . This has allowed the determination of the K_i values of both drugs for inhibition of swelling, using the mathematical analysis for inhibition by a very-tight-binding inhibitor as described previously [8]. Although this analysis is capable of deriving best-fit values for both the K_i and the number of inhibitor-binding sites, we chose to fix the latter at 120 pmol/mg of mitochondrial protein, the value calculated in earlier studies [8]. This mathematical constraint allows a more accurate comparison of the relative K_i values of cyclosporins A and G. Data from several experiments with both heart and liver mitochondria are summarized in Table 1. It is clear that the K_i for cyclosporin G is 10–20-fold higher than that for cyclosporin A in both cases.

Effects of cyclosporin analogues on mitochondrial PPIase

In parallel with the studies on the inhibition of Ca^{2+} -induced swelling by cyclosporin analogues, we have investigated their relative potency as inhibitors of mitochondrial matrix PPIase. This enzyme is assayed spectrophotometrically by measuring the rate of hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-nitroanilide by excess chymotrypsin, which only acts on that fraction of the peptide (about 87%) with a *trans* peptide bond between Pro and Phe [5,17]. After the initial burst of hydrolysis of the *trans* isomer and release of the nitroanilide product, subsequent hydrolysis is limited by the slow rate of *cis*-*trans* isomerization of the remaining *cis* peptide. It is this that is stimulated by PPIase. In Fig. 2(a) we show the time courses of peptide hydrolysis in the presence and absence of isomerase or of different cyclosporin analogues or FK-506. Direct measurement of initial rates of hydrolysis are difficult because of the very large and rapid burst phase. However, subsequent hydrolysis is pseudo-first-order, and can be linearized by using a log plot [14,17], as shown in Fig. 2(b). The slope of this plot gives the rate constant, which can be used to estimate accurately the initial rate of isomerization. In agreement with our earlier studies [8,16], it is clear from Fig. 2(b) that 500 nM-cyclosporin A inhibited the isomerase totally, whereas 500 nM-FK-506 was without effect (traces vii and ii). Even at 5 μM no inhibition by FK-506 was observed (results not shown). Cyclosporin G gave almost total inhibition at 500 nM (trace vi), but cyclosporin H had little effect (< 20%) at this concentration (trace iii). However, when added at 20 nM it became clear that cyclosporin A was a more effective inhibitor than was cyclosporin G (traces iv and v). A more detailed analysis was performed using a range of inhibitor concentrations in order to derive K_i values as described above. In this instance the total number of binding sites was fixed at 400 pmol/mg of matrix protein for both heart and liver mitochondria [8]. Derived K_i values from several experiments are given in Table 1. Once again, it is clear that the K_i value for cyclosporin G is about 10-fold higher than that for cyclosporin A.

DISCUSSION

PPIase is involved in pore formation

In this paper we have shown that the inhibitory potency of three cyclosporin analogues on mitochondrial PPIase matches their ability to inhibit opening of the non-specific permeability pathway of mitochondria exposed to supraphysiological Ca^{2+} concentrations. The concentration of binding sites for cyclosporin A inhibition of the non-specific permeability pathway is also similar to the concentration of the isomerase within the matrix [8]. These observations confirm our previous conclusion

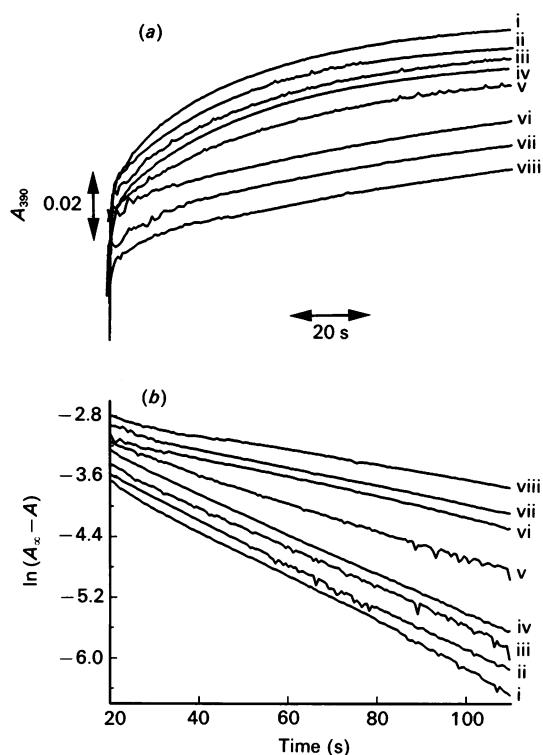


Fig. 2. Effect of cyclosporin (Cy) analogues and FK-506 on the activity of PPIase

Details of the assay are given in the Experimental section. Liver mitochondrial matrix fraction (26 μg of protein/ml) was present in traces i-vii with the following additions: none (i), 500 nM-FK-506 (ii), 500 nM-CyH (iii), 20 nM-CyG (iv), 20 nM-CyA (v), 500 nM-CyG (vi) and 500 nM-CyA (vii). The reaction in the absence of matrix extract is shown in trace viii. In (a) a direct display of absorbance is shown, whereas in (b) the data are linearized, assuming first-order kinetics [14,17].

that the isomerase is responsible for the opening of the non-specific pore in the mitochondrial inner membrane. The novel immunosuppressant FK-506 inhibits neither the matrix PPIase nor the pore opening. However, it is known that this drug inhibits a cytosolic isoenzyme of PPIase [12-16].

It is noticeable that the K_i values for inhibition of mitochondrial swelling were about 5-fold higher than for the inhibition of the isomerase. The equation used to calculate the K_i values assumes that the inhibitor is non-competitive, but is also appropriate for a competitive inhibitor acting in the presence of a substrate at a concentration substantially below its K_m [14]. Our assay of the matrix PPIase utilizes a small artificial peptide substrate present at a concentration well below its K_m [5,14], and thus our analysis will produce an accurate estimate of the K_i for cyclosporin. We are unable to assay the enzyme at higher substrate concentrations, closer to the K_m value, since the hydrolysis of the *trans* isomer of the peptide would produce too large an initial absorbance change. Thus direct assessment of the nature of the inhibition is not possible. If the inhibition is competitive and the natural protein substrate within the mitochondrial matrix binds substantially more tightly to the enzyme than does the artificial peptide, derived K_i values in the swelling experiments could be higher than the real values. This would only occur if the natural substrate was present at concentrations similar to, or above, its K_m . Another plausible explanation for the difference in the K_i values would be that the cyclosporin concentration within the matrix is less than that added outside, perhaps as a consequence

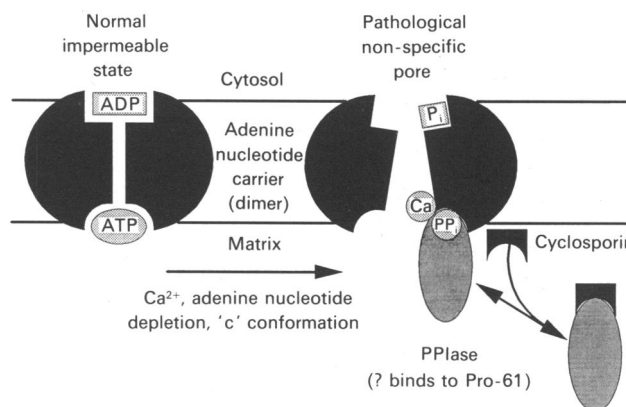


Fig. 3. Scheme to illustrate the proposed mechanism of mitochondrial pore opening

of any membrane potential or pH gradient existing across the mitochondrial inner membrane under the conditions used to assay swelling.

We have proposed that the isomerase causes pore opening through an interaction with the adenine nucleotide translocase [8], as shown diagrammatically in Fig. 3. In summary, our hypothesis is based on the observation that Ca^{2+} -induced swelling may also be inhibited by bongkreikic acid and ADP, which stabilize the 'm' conformation of the carrier, while carboxyatractyloside, which stabilizes the 'c' conformation, enhances swelling [8-11]. The c conformation is believed to have a proline residue (Pro-61) that is exposed to the matrix surface, but this residue is hidden in the m conformation [18,19]. There is also evidence that matrix Ca^{2+} can bind to the carrier in the c conformation [8]. Thus we believe that in the presence of Ca^{2+} , and with no adenine nucleotides bound to the carrier, PPIase binds to Pro-61 and causes a conformational change sufficient to form a non-specific pore.

The importance of proline residues in conformational changes of membrane proteins has been suggested by others [20-22]. For the opening of the non-specific pore it is probable that a conformational change associated with *cis-trans* isomerization of a proline peptide bond is only maintained so long as the isomerase remains bound to a proline on the carrier. The closing of the pore is very rapid upon removal of the enzyme by cyclosporin addition [8]. This would not be expected if non-enzymic isomerization was responsible for the restoration of the original conformation. Reversible conversion of a specific antiporter into a non-specific pore can be achieved for the purified mitochondrial aspartate/glutamate carrier by treatment with low concentrations of organomercurials [23]. This demonstrates the feasibility of a specific carrier changing its conformation to become a non-specific pore, but direct proof must await reconstitution of the carrier and isomerase into proteoliposomes.

Implications for the immunosuppressive and toxic effects of cyclosporin analogues

PPIases are ubiquitous and may represent a family of proteins [2,12-14,24]. It has been suggested that they might play a role in protein folding during synthesis, but this is unlikely in view of the quite rapid spontaneous rate of isomerization. The notion that they may bind to proline residues of existing proteins in the presence of Ca^{2+} to cause conformational changes, and thus modulate enzyme or carrier activity, is an attractive one. Indeed, the visual transduction pathway of *Drosophila* is known to involve conformational changes of visual pigments and to require

a protein with strong structural similarity to cyclophilin [25]. Thus the mitochondrial effects of cyclosporin may provide a model system for the study of such an interaction, and so provide new insights into the cyclosporin-sensitive signal transduction pathway involved in the activation of T-lymphocytes. It is also possible that the inhibition of mitochondrial PPIase is responsible for the toxic effects of cyclosporin A on liver and kidney [1,2]. In support of this hypothesis, toxicity is associated with changes in mitochondrial morphology [1,26] which are not as severe with cyclosporin G as with cyclosporin A [27,28]. FK-506 appears to lack these side-effects [29]. We have provided evidence that the non-specific permeability pathway can open in normal healthy liver cells stimulated with hormones [8,30]. Others have shown that sucrose, which is normally unable to enter mitochondria *in vitro*, can enter the mitochondrial matrix *in situ* [31]. Fluorescent microscopic studies of cells stained with Rhodamine G to report mitochondrial membrane potential show that mitochondria may undergo occasional depolarization, consistent with pore opening [32]. Thus we may speculate that occasional pore opening is essential for the well-being of mitochondria, perhaps to release by-products of metabolism for which no specific transport pathways exist. Permanent closure of the pore with cyclosporin treatment would lead to the build-up of such metabolic waste products, to the detriment of the mitochondria.

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